RESEARCH PLAN PROPOSAL

Evaluation of White Rot Fungal Cultures for Biopulping of Ankra (*Calotropis procera*) Fiber

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INTRODUCTION

Paper has been an essential part of our civilization for at least 2000 years and perhaps, because of our familiarity with it, we do not tend to think of it as a particularly complex material. We use it so commonly in our day to day life that we consider it just as a simple commodity material and we take paper for granted. However, paper is actually derived from plant sources and therefore has morphological as well as physical and chemical complexity (Chauhan, 2009a).Paper consists of a web of pulp fibers derived from wood or other plants from which lignin and other non-cellulose components are separated by cooking with chemicals at high temperature. In the final stages of papermaking, aqueous slurry of fiber components and additives is deposited on a wire screen and water is removed by gravity, pressing, suction and evaporation (Biermann, 1993).

The pulp and paper industry is dominated by North American (United States and Canada), Northern European (Finland, Sweden, and North-West Russia) and East Asian countries (such as East Siberian Russia, China, Japan, and South Korea). China is the world's leading producer of paper and the United States behind it (Mike, 2013). In India, the paper industry is a combination of small, medium and large mills. Paper industry in India is the largest industry and stood on twentieth position among the world's paper producing industry (Kesalkar *et al*., 2012). According to the Indian Paper Manufacturers Association (IPMA), Indian paper industry accounts for about 1.6% of the world's production of paper and paper board.

The production of paper and its use has a number of adverse effects on the environment which are collectively known as Paper Pollution. Pulp and paper industry is the third largest industrial polluter to air, water and land (deforestation) and releases over 100 million kg of toxic pollution each year (Kesalkar *et al*., 2012).Worldwide the pulp and paper industry is the largest consumer of energy and accounts for 4% of the entire world's energy use. This industry requires substantial quantities of water to produce a ton of product than any other industry (Pokhrel and Viraraghavan, 2004; Iqbal *et al*., 2013). The quantity of water consumed per ton of paper is 250 cubic meters for mill paper while 150 cubic meters for handmade paper (Ibramanian and Kumar, 1993). The generation of waste water per metric ton of paper produced depends upon the nature of raw materials used, process (chemical or biological), technology used and amount of water used. The waste water of the industry contains high level of Chemical Oxygen Demand (COD), Biological Oxygen Demand

(BOD), chlorinated compounds, fatty acids, tannins, resin acid, sulphur and sulphur compound, lignins, chlorinated phenols, guaiacols, catechols, furans, dioxins, aliphatic hydrocarbons etc.Many of these pollutantsare known to be toxic, mutagenic, persistent, and bio-accumulating(Bajpai, 1999).Thepaper industry also causes air pollution through the discharge of gaseous pollutants involving a variable mixture of Hydrogen Sulfide, Methyl Mercaptans, Dimethyl Sulphide and Dimethyl Disulphide. (Dey& Gupta, 1992).

The environmental impact of pulping and bleaching plant effluents has recently gained serious attention by the pulp and paper industry. In the last 10 years or so, there has been a monumental research seeking to improve pulping and bleaching processes as well as their products. Biological methods have shown a new ray of hope in this regard.The general use of alternative, environmental friendly technologies that introduce lignocellulose degrading enzymes at different stages of pulp and paper manufacture as a pretreatment to pulping (biopulping), bleaching (biobleaching) or wastewater treatment has allowed considerable savings in resources and a reduction of pollutants in the waste water (Perez *et al*., 2002).

Biopulping is the treatment of wood or other lignocellulosics with lignin degrading fungi prior to pulping, so that the cellulose fiber gets free and used for making paper(Iqbal *et al*., 2013, Singh *et al*., 2010). White rot fungi or basidiomycetes are among the most extensive degraders of lignocellulosic biomass. The lignin degrading enzymes secreted by these fungi target selectively on the lignin thereby leaving fibrous residue (Lobos *et al*., 2001, Fackler *et al*., 2007, Husaini *et al*., 2011). Various white rot fungal cultures (*Phaenerochaete chrysosporium, Ceriporiopsis subvermispora, Phlebia radiata, Coriolus versicolar, Pleurotus eyngii, Pycnoporus cinnabarinus* etc.) have been reported to be suitable for the process of biopulping which results in significant energy savings during refining and improvement of paper strength properties besides reducing environmental impact of the industry (Kirk *et al.*, 1994; Scott *et al*., 2002; Singh *et al*., 2010).

Therefore, it seems important to isolate suitable cultures of selective lignin degrading white rot fungi for evaluating their potential of biopulping in the lignocellulosic raw materials particularly the non-woody fibers so as to reduce pollution levels of the paper industry and to conserve forest resources besides saving energy and chemicals.

REVIEW OF LITERATURE

Lignocellulose: The Raw Material for Papermaking

Lignocelluloses are a renewable organic material and are the major structural component of all plants. Lignocelluloses make up about half of the matter produced by photosynthesis. Because of their renewable nature, they have gained a special importance for product development (Asgher *et al*., 2013) like paper and biofuels. (Isroi *et al*., 2011). Lignocellulose is a heteropolymer consisting mainly of three components,cellulose, hemicellulose and lignin (Fengel and Wegener, 1989; Eaton and Hale, 1993) that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions (Betts *et al.,* 1991).Typical chemical composition of various lignocellulosic materials has been shown in table-1. Chemical composition of any lignocellulosic material gives an idea of how feasible it is as a raw material for papermaking.

Table-1: TypicalChemical Composition of Lignocellulosic Materials (Betts *et al*., 1991)

Raw material	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Hardwoods	18-25	45-55	24-40
Softwoods	25-35	45-50	25-35
Grasses	10-30	25-40	25-50

Cellulose is the principal component of cell wall and of fibers. It is a linear polymer of glucose linked through α 1,4-linkages and is usually arranged in microcrystalline structure. The degree of polymerization (DP) of cellulose chains range from 500 to 25,000 (Kuhad *et al*., 1997; Leschine,1995). Hemicellulose is a heteropolysachharide composed of different hexoses, pentoses and glucoronic acid. It has more solubility than cellulose and is frequently branched with short branched chains of hexoses, e.g. mannose units in mannans and pentoses such as xylose units in xylans. (Kuhad *et al*., 1997). Lignin is regarded as a complex polymer of variously linked aromatic alcohols (coniferyl, sinapyl, and p-coumaryl alcohol) and is further linked to hemicelluloses and cellulose. Lignin forms a physical seal around the cellulose and hemicelluloses (Sanchez, 2009). There are no chains like cellulose or hemicelluloses which makes its enzymatic hydrolysis extremely difficult. In pulp and paper

industry, cellulose is needed further for paper making, so removal of lignin is required to get or to free cellulose from any lignocellulosic raw material.

Fiber Properties of Papermaking Raw Material

The fiber properties of the lignocellulosic raw materials are an important factor which affects the quality and use of the paper.For fine papers, both long and short fibers are needed. The long fibres from softwoods (coniferous trees, fiber length 2-5 mm) or from non-woody species such as flax (*Linum usitatissimum L.*), hemp (*Cannabis sativa L.*) and kenaf (*Hibiscus cannabinus L.*), of fiber length 28 mm, 20mm and 2.7 mm respectively, form a strong matrix in the paper sheet. The hardwood fibers (0.6-1.9 mm) or grass fibres (0.7mm) are shorter in length and contributes to the pulp properties like opacity, printability and stiffness (Saijonkari-Pahkala, 2001 and Hurter, 1988). The plant families *Gramineae, Leguminosea* and *Malvaceae* are the promising non woody species for fibre production (Nieschlag *et al*., 1960). Presently, the grasses and monocotyledons as well as flax and hemp grab a more attention and focus, as a raw material in pulp and paper industry (Kordsachia *et al*., 1992).The taxonomy of fiber plants is shown in figure 1. According to the Paavilainen, 1996, in India and China over 70% of raw material used by the pulp and paper industry comes from non-woody plants and agricultural residues.

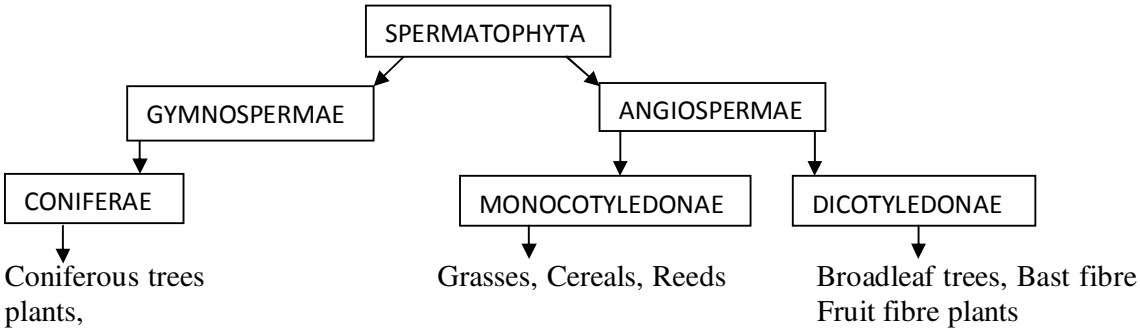


Figure 1: The taxonomy of fibre plants (Ilvessalo-Pfaffli, 1995).

According to Pandey and Gupta (2003), the plant fibers are classified mainly on the basis of morphological nature, structure, origin and uses. Based on botanical origin, vegetable fibers excluding wood fibers are grouped into soft fibres/bast fibers (fibers from the bast, or bark of some nonwood plants are used for papermaking (Chandra, 1998), hard fibers or structural fibers. The plant fibers are extracted from different parts such as stem, leaf, petioles, roots, fruit and seeds.

Bast Fibers

Bast fibers refer to all fibers obtained from the phloem of the vascular tissues of dicotyledons (Saijonkari-Pahkala, 2001). Bast fibers are exogenous in origin and are generally more durable, stronger, soft, and smooth than structural fibers. They are associated with vascular tissues, such as phloem, pericycle and cortex. Example of bast fibers are *Corchorus oliotorus* (Jute), *Cannabis sativa* (Hemp), *Linum usitatissimum* (Flax), *Hibiscus sabdariffa* (Roselle), *Boehmeria nivea* (Ramie), *Calotropis procera* (Ankara) etc. Bast fibers are commercially exploited for items like gunny bags, ropes, fishing nets, textiles etc. When these get old and cannot be used for their original purpose, they serve as a source of non-wood raw material for the paper industry (Chandra, 1998). The member of the families *Urticaceae, Lineacea, Asclepiadaceae, Moraceae* are known to yield bast fibers. The bast fibers are extracted by retting (a microbial process that breaks the chemical bonds holding the stem together and thereby allowing separation of the bast fibres from the woody core). Bast fibers are used as raw material for making paper when strength, permanence and other special properties are needed. (Atchison 1987, Ilvessalo-Pfaffli, 1995).

Lignin Removal for Papermaking (Pulping)

Lignin removal is an important technical issue for paper manufacturing According to Lobos *et al*. (2001) lignin is a highly irregular biopolymer with an undefined structure. It is composed of oxygenated phenyl-propanoid units which are linked among various types of C-C and C-O-C bonds. Because of these unusual features, this macromolecule is rather difficult to biodegradation. However, white rot fungus is the only group of microorganisms which have the capacity to break down lignin extensively. Therate of delignification varies considerably depending upon the type of fungi and relative rates of attack on lignin and carbohydrates in raw materials (Blanchette, 2000).Biomass recalcitrance towards enzymatic hydrolysis is correlated with the content and composition of lignin (Studer*et al*., 2011). Physical, chemical and biological pretreatments, or combinations of these processes, have been reported for deconstructing lignocellulosic biomass and removing lignin (Alvira *et al*., 2010; Yu *et al*., 2011). Biological pretreatment has received particular interest due to greater specificity, lower energyrequirements, lower pollution generation and higher yields of desired products.

Microbes in Lignin Degradation

A number of microorganisms are involved in the degradation of lignocellulosic materials in natural environment however some fungi especially white rot have developed the necessary enzymes to break lignin apart. Mixed cultures of fungi, actinomycetes and bacteria in soil and compost can also mineralize lignin (Tuomela *et al*., 2000).

Bacteria and Actinomycetes:The *Streptomycetes species* have been shown to degrade low levels of lignin (Crawford, 1978 and Watanabe *et al*., 2003).Bacteria are generally considered as secondary lignocellulose degraders which can degrade cellulose and hemicelluloses both aerobically as well as anaerobically (Walker and Wilson, 1991). *Thermobifida fusca* and *Cellulomonas composti* are the example of aerobic hemicelluloses degraders (Baguin and Aubert, 1994) while *C. thermocellum* and *Clostridium cellulolyticum* are among the best-studied anaerobic, cellulose-degrading bacteria (Bayer *et al*., 2008). However, the evidence of bacterial lignin degradation is sparse. Actinomycetes can also decompose lignin, but typically degrade less than 20% of the total lignin present (Cawford, 1986; Basaglia etal, 1992). According to Trigo and Ball (1994), actinomycetes play a significant role in the humification process associated with soils and composts. They produce extracellular peroxidases e.g. lignin peroxidase-type enzyme (Pasti *et al.,* 1991; Mercer *et al*., 1996).

Fungi:Fungi are the primary degraders of lignocelluloses (Rabinovich *et al*., 2002; Sanchez 2009). In addition to secreting enzymes that are critical to lignocelluloses decomposition, fungal growth on lignocelluloses is promoted by the formation of mycelia that allow filamentous fungi to transport nutrients, including nitrogen and iron, to the carbon rich lignocellulosic substrate (Hammel, 1997). Many fungi are also more resistant to wood-derived biocides that limit bacterial growth. These compounds include tannins and various phenolic compounds (terpenes, sitilbenes, flavanoids and tropolones that are particularly abundant in the heartwood of fallen trees. The majority of fungi that degrade wood have been characterized as the member of the phylum Basidiomycota and are characterized by either brown-rot or white-rot fungus decay.

Brown Rot Fungi: Brown-rot fungi mainly decompose the cellulose and hemicellulose components in wood, but they can also modify the lignin to a limited extent (Eriksson *et al*., 1990). The decayed woods are typically characterized by dark, reddish brown color and dry, crumbly and brittle consistency (Martinez *et al*., 2005), shrink, and typically broken into brick-shaped or cubical fragments that easily break down into brown powder. The brown color indicates the presence of modified lignin in wood (Blanchette, 1995).

White Rot Fungi*:* Lignin degradingbasidiomycetes, collectively known as white rot fungi are the only microbes that have been convincingly shown to efficiently depolymerize, degrade and mineralize all components of plant cell walls including cellulose, hemicelluloses and the more recalcitrant lignin (Kersten and Cullen, 2007). However, white-rot fungi successively depolymerize cell wall carbohydrates only to the extent that utilize hydrolysis products in their metabolism. There are more than 10,000 different species of white rot fungi in nature with varying capacities to degrade lignin, cellulose and hemicelluloses (Kang *et al*., 2007). According to Tuomela *et al*. (2000), and Singh *et al.* (2010) white rot fungi are very common to those present in compost, rotten wood, saw dust, and different forest waste or soil environment where lignin substances are decomposed under the natural conditions.

Patterns of Lignin Degradation by White Rot Fungi

White rot fungi degrade lignin with two modes of action namely selective and non-selective decays (Blanchette, 1995). Selectivity of white rot fungi regarding lignin degradation depends on the lignocellulosic species (Hakala *et al*., 2004), cultivation time and other factors (Hattakka and Hammel, 2010).

Selective decay: This includes selective degradation of lignin and hemicellulose fractions while the cellulose fraction is essentially unaffected. Examples of white rot that possess selective decay are*Ceriporiopsis subvermispora, Dichomitus squalens, Phanerchaete chrysosporium and Phlebia radiate*(Mahajan, 2011).

Non-selective decay: This includes degradation of approximately equal amounts of all fractions of lignocelluloses. Examples of white rot that possess non-selective decay are *Trametes versicolor* and *Fomes fomentarius* (Mahajan, 2011).

Fungal Enzymes Involved in Lignin Degradation

Lobos *et al.* (2001), Kirk and Faerell, (1987) reported that the white rot fungi produces a comprehensive and unique set of oxidative enzymes termed as lignin modifying enzymes (LMEs). LMEs include mainly two ligninolytic enzyme families: i) Phenol oxidase (laccase) and ii) Heme peroxidases (LiP, MnP and VP). (Dashtban *et al*., 2010). Main features of the two groups of enzymes are given in table-2. These fungi have an ability to decay lignin directly in natural environment and to oxidize or degrade lignin in vitro. (Fackler*et al*.,2007). Therefore, lot of interest has been developing towards using mycological techniques in various industrial applications particularly in the paper industry.Almost all white rot fungi

produce MnP and Laccase but only some of them produce lignin peroxidase. Lignin degradation by white-rot fungi is an oxidative process (Kuhad *et al.*, 1997; Leonowicz *et al.*, 1999).

Table-2: Ligninolytic enzymes involved in lignin degradation (Hatakka 2001, Dashtban *et al.*, 2010)

Enzyme	Reaction	Metals or Ions	Cofactor or substrate, "Mediator"	Main effect or reaction
Lignin peroxidase, LiP	1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 = 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H_2O	Iron	H_2O_2, Veratryl alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase, MnP	2Mn(II) + $2H^+$ + H_2O_2 = 2Mn(III) + $_2H_2O$	Ca^{2+} Cd^{2+} Mn^{2+} Sm^3	H_2O_2, Mn, organic acid as chelator, thiols, unsaturated lipids	Mn(II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxy; other reactions in the presence of additional compounds
Versatile peroxidases, VP (hybrid peroxidase)	donor + H_2O_2 = oxidized donor + $_2H_2O$ (e.g. reactive black 5 + H_2O_2 = oxidized reactive black 5 + $2H_2O$)	Mn^{2+} Ca^{2+} Cu^{2+} Iron	H_2O_2, same or similar compounds as for LiP and MnP	Same effect on aromatic and phenolic compounds as LiP and MnP
Laccases, Lac	4 benzenediol + O_2 = 4 benzosemiquinone +2 H_2O	Ca^{2+} Cd^{2+} Cu^{2+} H_2O_2 Imidazole K^+	Oxygen; mediators eg. Hydroxybenzotriazole or ABTS	Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators

Utilization of White Rot Fungi for Biopulping

The biodegradative abilities of white rot fungi are remarkable, both in terms of the number of different chemicals that can be oxidized and in the nature of chemicals that can be oxidized. Their biodegradation system is versatile. Lignin degradation by white rot fungi especially those that selectively degrade lignin from wood is a characteristic that makes them ideally suitable for industrial applications like paper industry where lignin or various phenolic compounds must be altered or removed (Kulshreshtha *et al.*, 2013; Maijala *et al.*, 2008; Fackler *et al.*, 2007; Lobos *et al.*, 2001 and Blanchette *et al.*, 1987).Lawson and Still (1957) at the West Virginia Pulp and Paper Company (now Westvaco Corporation) were the first who considered the use of white-rot fungi for the biological delignification of wood. Having realized the great potential ofwhite rot fungi in selective lignin degradation, two sequential biopulping consortia have conducted extensive research on biopulping (Kirk *et al.*,1994) and reported the process to be highly beneficial with various types of fibers/lignocellulosic raw materials used in papermaking. The potential uses of white rot fungi for biopulping, biobleaching and treatment of pulp mill waste effluents have been reviewed (Kulshrestha *et al.*, 2013). Till now a number of researchers have worked on biopulping and suggested a number of fungi capable of degrading lignin which may have number of applications in pulp and paper industry as given in the table-3.From the table-3, it is obvious that the process of biopulping has been worked out on a range of lignocellulosic raw materials including woody as well as non-woody plants. However, almost all the work has been conducted from the view point of mill papers but the lignocellulosic raw materials used in making specialty papers viz. handmade papers have not been explored although it may be much more useful for the sector as the developed technologies may be applied much more easily in the handmade paper sector than the conventional paper industry (mill-sector) due to the utilization of batch procedures at small scales in the handmade paper sector (Chauhan *et al.*, 2009). The handmade paper has several excellent qualities when contrasted with those of mill-made paper. It is elegant in appearance and provides an exquisite surface for writing. It has good strength and is practically indestructible; it actually has double the strength of mill-made paper and does not turn brittle with age. It can be used for several artistic and decorative purposes. For these reasons, it fetches a better price than mill made paper.The Indian handmade paper sector has been confronting with various issues including the scarce availability and cost prohibitiveness of the cotton hosiery waste, being used traditionally as

the major raw material, inconsistent quality and cost effective production of Handmade paper.

Table 3: Raw Materials & Fungal Cultures Used for Biopulping By Various Researchers.

RAW MATERIALS	FUNGAL SP.	REFERENCES
Mangnifera indica	*Flavadon flavus, Schizophyllum commune*	Padihar and Albert, 2012
Populus tremuloides	*Antrodiella sp. (RK-1)*	Patel *et al*., 1994
Spruce wood	*Ceriporiopsis subvermispora, D. squaleus, P. trematous, P. radiata*	Fackler *et al*., 2007
Abies balsawes, Acer sacchrum, Alnus rubra, Beluta papyrifera, Picea mariana, Pinus strobes, Pinus strobes, P. resiuola, Populus tremeloida, Tolic americana	*Coriolus versicolor, P. pini, Poria medulla, Scytinostroma galactum, S. galactinum, D. squalans, P. tremelloses*	Blanchette *et al*., 1998
Pinus dens flora, Populus alba, X. glandulosa	*P. chrysosporium*	Kang *et al*., 2007
Radiata pine	*T. versicolor, C. subvermispora, P. chrysosporium*	Lobos *et al*., 2001
Paddy straw	*Pleurotus eous*	Koshy *et al*., 2011
Sugarcane bagasse (*Saccharum officinarum*)	*P. chrysosporium, K. aerogenes*	Jha *et al*., 2002
Banana fruit stalk	*Coriouls versicolor, Phanerochaete chrysosporium*	Helmy and Meligi, 2002
Wheat straw	*Fusarium concolor*	Li *et al*., 2008
Pineapple leaf	*Ganoderma lucidum*	Hariharan *et al*., 2013
Rice bran	*Trichoderma spp*.	Desai *et al*., 2011

Rags are traditionally used principle raw material for making handmade paper but with the growing demand of handmade papers, the availability of rags is getting limited therefore the adoption and utilization of alternative raw materials is getting impetus. With the extensive R & D efforts carried out at KNHPI, Jaipur; it has been found that a number of ligno-cellulosic raw materials available as waste biomass in different parts of the country viz. Banana waste leaves/fiber, Pineapple fiber and leaves (*Ananas comosus*), Sunn Hemp (*Crotolaria*), Jute (*Corchorus capsularis*), Bhimal (Grewia oppositifolia) and Ankara (Calotropis procera),

certain forest weeds like Bodha grass (*Chloroxylon coloratus*) or Mentha (*Mentha piperita*), non-woody agro residues like straws, recycled waste paper and shredded currency waste of Reserve Bank of India, etc. have been identified as alternative raw materials for making handmade paper (Chauhan *et al*., 2009b).

Ankra Fiber *(Calotropis procera)*

Calotropis procera is an excellent raw material for the manufacture of specialty papers. Its long fiber length and high strength makes it a superior material for the production of thin lightweight papers of high porosity and excellent tear burst and tensile strengths (Peralta, 1996). It has special properties for making strong products like tea bags, large sausage casings, currency paper, cigarette and filter paper, and specialty products that require high wet strength combined with high porosity (Chandra, 1998).*Calotropis procera* (Ankra) has proved to be potential lignocellulosic raw material finding usefulness for making specialty handmade paper and products.Bioretting process has also been reported to be extremely useful for extracting good quality bast fiber from Ankra plant (Chauhan *et al*., 2013).

According to the Sharma*et al.* (2011)*Calotropis procera* is drought-resistant, salt-tolerant to a relatively high degree, and it disperses seeds through wind and animals. It quickly becomes established as a weed along degraded roadsides, lagoon edges and in overgrazed native pastures. *Calotropis procera* is native to India, Pakistan, Nepal, Afghanistan, Algeria, Iran, Iraq, Israel, Kenya, Kuwait, Niger, Nigeria, Oman, Saudi Arabia, United Arab Emirates, Vietnam, Yemen and Zimbabwe.It is available in Haryana, Punjab, Gujarat, Bihar and Rajasthan and widely available in Northwest India. At Rajasthan, it mainly occurs in the desert areas of Bikaner, Jodhpur, Pali, Jhunjhunu, Jaisalmer and Barmer.

Morphologically the plant is erect, tall, large, much branched and perennial shrub or small tree that grows on a height of 5.4m, with milky latex throughout. Bark is soft and corky, branches stout, leaves sub sessile, opposite, decussate, broadly ovate, oblong, elliptic or obovate, acute, thick, glaceous, green coloured with fine cottony pubescent hair on young. (Dwivedi *et al*., 2010). The Anatomical characteristics of its wood are given in table-4. (UNDP and Technical Paper). Its scientific classification is given below:

Scientific Classification (Sharma *et al*., 2011)

Kingdom:	*Plantae* – Plants
Subkingdom:	*Tracheobionta* – Vascular plants

Super division: *Spermatophyta* – Seed plants
Division: *Magnoliophyta* – Flowering plants
Class: *Magnoliopsida* – Dicotyledons
Subclass: *Asteridae*
Order: *Gentianales*
Family: *Asclepiadaceae* – Milkweed family
Genus: *Calotropis* R. Br. – calotropis
Species: *Calotropis procera*

Table 4: Average values of wood anatomical characteristics for *Calotropis procera*.

Items	Fiber length	Vessels	Fiber Diameter
Average	17.52-27.5 mm	15.0 /mm2	15-35 micron

Chemical Composition: Chemical composition of wood and any lignocellulosic material profoundly influences its utilization for pulp and particleboard industries, chemical feedstock and energy. The cellulose and lignin of *Calotropis* are stable chemical components and they did not vary between trees or between branches within trees. A chemical composition of *Calotropis procera* wood as reported are shown in table-5.

Table 5: The chemical composition of *Calotropis procera* wood (Pant *et al*. 2005).

Ash (%)	Extraction (%)	Lignin (%)	Holocellulose (%)	Solubility (%) in	
				Hot H_2O	1% NaOH
2	0.2	6	81.8	7.5	17.5

Keeping in view of the significance of biopulping process for the paper industry particularly the handmade paper industry and the potential of Ankra fiber for making good quality handmade papers, the present study is being proposed to isolate suitable strains of white rot fungi from natural habitats and to evaluate their effect on lignin degradation of Ankra fiber with a comparison from the standard cultures of white rot fungi procured from IMTECH, Chandigarh.

OBJECTIVES OF RESEARCH

- Isolation of white rot fungi from suitable solid and/or liquid samples.
- Screening of the potential ligninolytic white rot fungi.
- To evaluate the effect of isolated cultures on lignin degradation of non-woody lignocellulosic raw materials viz. bast fiber of *Calotropis procera*(Ankra).
- Comparison of their effect with those of the standard white rot fungal cultures procured from IMTECH, Chandigarh.
- Identification of the most suitable fungal culture that can be utilized further for biopulping of Ankra fiber.

METHODOLOGY

I. Sample Collection

Various solid samples including the lignocellulose rich compost piles, rotten wood samples, decaying twigs of Ankra plant anddecaying pulp samples, fruiting bodies from woody substrate and/or soil surface will be collected in zip-lock polythene bags. Liquid samples will be collected in the form of waste water of pulping process and effluents from paper industry in sterilized bottles with lid.

II. Isolation Of Fungal Cultures From The Samples Collected

Fungi will be isolated by placing the small pieces of solid samples on the centre of Potato Dextrose Agar (PDA) plates containing 0.01% (w/v) of chloramphenicol and chlorotetracycline in order to inhibit the growth of bacteria and 1% (w/v) of Benomyl, a benzimidazole fungicide to select for wood decay fungi after surface sterilization with 95% ethanol. The inoculated plates will be incubated at 30±2 ^{0}C until the mycelium develops. The fungal cultures grown will be subjected to repeated sub-culturing on fresh agar plates until pure colonies are obtained, (Fusaro, 1972).

III. Screening Of the Potential Ligninolytic Fungi

The isolated white rot fungal cultures will be cultivated on solid media containing indicator compounds that enable the detection of laccases and other ligninolytic enzymes as specific colour reactions. Different indicator compounds viz. 0.01% Guaiacol, 0.1% ABTS, 0.04% Poly R-478, 0.04% (w/v) RBBR , and 0.5% Tannic acid will be added and inoculated plates will be observed daily during incubation for the following changes:

- The formation of reddish brown halo in guaiacol supplemented plates and dark-green halo in the ABTS supplemented plates indicates a positive laccase secretion. The diameter of the halo indicates the level of laccase produced (Gnanasalomi and Gnanadoss,2013).

- Decolorization of the polymeric dye Poly-R 478 (Sigma) by fungi positively correlates with production of the polyphenoloxidases lignin peroxidase, Mn dependent peroxidase (Boominathan and Reddy, 1992) and laccase (Pointing, 1999).

- Appearance of a brown oxidation zone around fungal colonies in the plates supplemented with Tannic acid gives an indication of overall polyphenoloxidase activity and is not specific to any one LME (Pointing, 1999).

IV. Microscopic Observation of the Fungal Isolates

Isolation of basidiomyceteous fungi will be confirmed by microscopic observation of septate hyphae and presence of clamp connections. (Silva *et al*., 1990; Ortiz *et al.* 2013).

V. Biological Pretreatment Of Ankra Fiber With Suitable Fungal Isolates And Standard Cultures

The chopped Ankra fiber will be subjected to biotreatment with the suitable isolates of white rot fungi and incubation at 30˚C for a period of 1-2 weeks (Husaini *et al.,* 2011; Nazarpour *et al*., 2013) at a relative humidity of around 65-70%.

VI. Enzyme Extraction and Their Assay

Biotreated Ankra fiberwill be extracted with 50 mM Sodium Acetate buffer (pH 5.5) to recover the extracellular enzymes produced during the fungal growth. (Husaini *et al*., 2011).Oxidative enzymes will be assayed with the following substrates and enzyme activities will be expressed as units per milliliter, where 1 U is defined as 1 millimole of substrate oxidized per min or otherwise stated.

- **Laccase Assay:**Laccase will be assayed using 1.0 mM DMP (2, 6-dimethoxyphenol) in 100mM Citric Acid buffer at pH 5.0 as substrate and noting the absorbance at 468nm (λ_{468}) (Husaini *et al*., 2010, Kiiskinen *et al.*,2004).

- **Manganese Peroxidases (MnP) Assay:** MnP activity will be assayed by the oxidation of 1.0 mM $MnSO_4$ with sodium tartrate buffer at pH 4.5. The

absorbance will be measured with UV-Vis spectroscopy λ_{238}. (Husaini *et al*., 2010, Chi *et al*., 2006).

- **Lignin Peroxidases (LiP) Assay:** This will be assayed by using Veratryl alcohol as substrate. Reactions will be carried out with 50mM Sodium Tartrate buffer at pH 2.5, enzyme extract, 2.0 mM Veratryl alcohol and 500 µM H_2O_2. The reactions will be monitored with UV-Vis spectroscopy at λ_{310}(Husaini *et al*., 2010, Chi *et al*., 2006).

VII. Evaluation of Fungal Treated Ankra fiber

- ***Weight loss*** will be determined after fungal treatment (Korean standard test method, Kang *et al*., 2007), calculated by the equation as follows:

  Weight loss (%) = $(W_1-W_2)/W_1$ X 100

  W1= oven dry weight of raw material before test.
  W2= oven dry weight of decayed raw material after test.
  The weight loss of raw material will increases with decay.

- ***Ash content***: This will be analyzed according to Oluwadare and Asagbra (2008) method.

- ***Analysis of Chemical Composition of Ankra fiber***

  - ***1% NaOH Solubility****:* According to TAPPI Test Methods T 212 om-02 (TAPPI, 2002).
  - ***Holocellulose Content*****:** This process will be done by TAPPI Standard Test Methods T 222 om-88.
  - ***α-Cellulose:*** will be determined using TAPPI Test Methods T 203 cm-99 (TAPPI, 1999).
  - ***Hemicellulose:*** The percentage hemicelluloses will be calculated by subtracting the percent α-cellulose from holocellulose (Nazarpour *et al*., 2013).
  - ***Lignin Content:*** Both acid soluble and insoluble lignin will be determined.
    - Acid soluble lignin (Klason lignin) will be determined according to TAPPI Test MethodsT-222 om-02 (TAPPI, 2002).

- Acid-insoluble lignin will be determined according to the TAPPI Test methods T 222 om-02 (TAPPI, 2002).

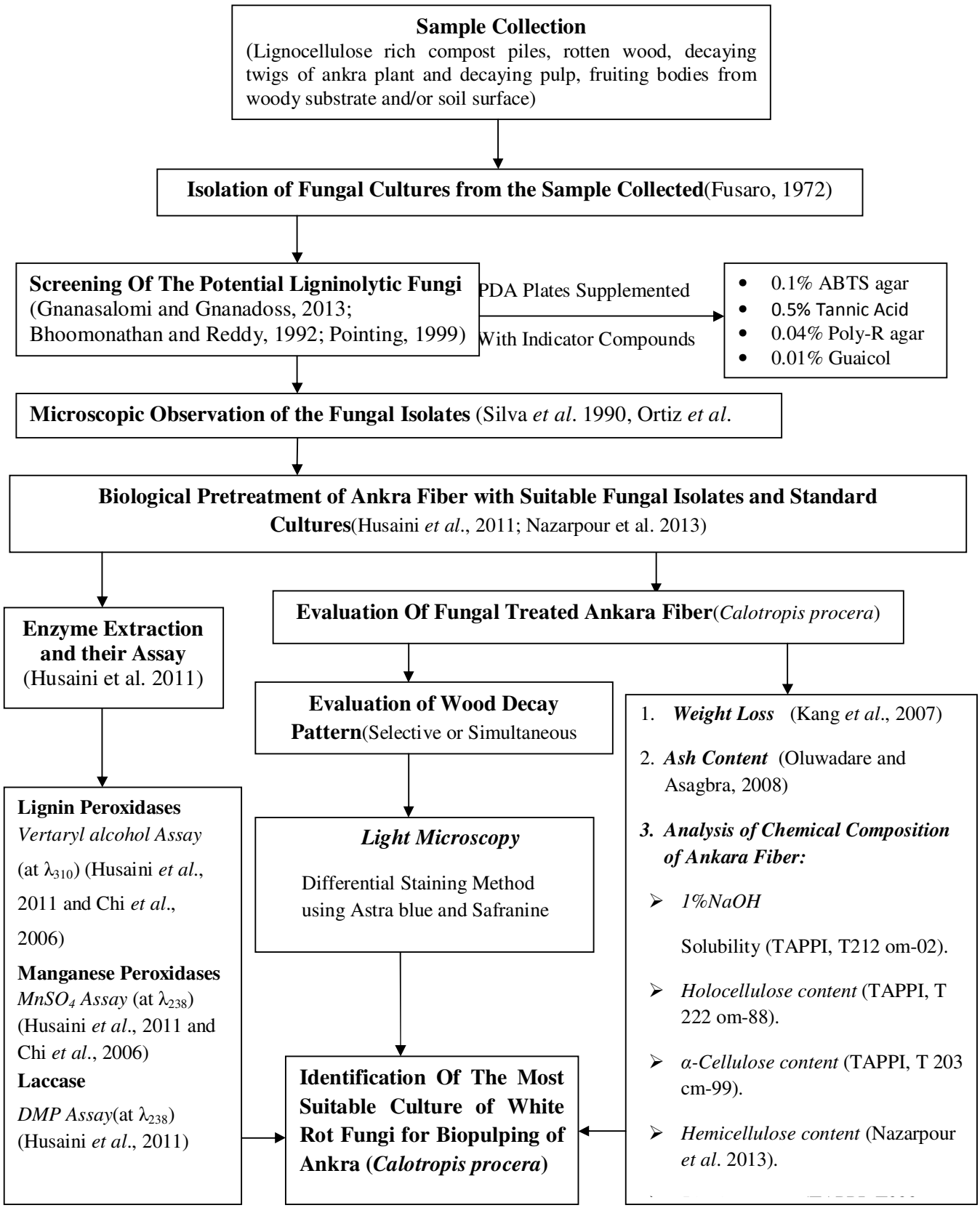
VIII. Evaluation of Wood Decay Pattern (**Selective or Simultaneous**).

The decay pattern of Ankra fiber will be evaluated with Light microscopy using a simple method of differential staining to assess the selectivity of delignification by white rot fungi. The two stains namely astra blueand safranine will be used as astra blue stains cellulose blue only in the absence of lignin while safranin stains lignin regardless of whether cellulose is present (Srebotnik and Messner, 1994).

IX. Identification of the Most Suitable Culture of White Rot Fungi for Biopulping of Ankra Fiber

Based on all the observations and combined inference, one or two cultures of white rot fungi will be identified as the most suitable ones for the biopulping of Ankra fiber.

PLAN OF WORK


Sample Collection
(Lignocellulose rich compost piles, rotten wood, decaying twigs of ankra plant and decaying pulp, fruiting bodies from woody substrate and/or soil surface)
Isolation of Fungal Cultures from the Sample Collected(Fusaro, 1972)
Screening Of The Potential Ligninolytic Fungi (Gnanasalomi and Gnanadoss, 2013; Bhoomonathan and Reddy, 1992; Pointing, 1999)
PDA Plates Supplemented With Indicator Compounds
0.1% ABTS agar
0.5% Tannic Acid
0.04% Poly-R agar
0.01% Guaicol
Microscopic Observation of the Fungal Isolates (Silva et al. 1990, Ortiz et al.
Biological Pretreatment of Ankra Fiber with Suitable Fungal Isolates and Standard Cultures(Husaini et al., 2011; Nazarpour et al. 2013)
Enzyme Extraction and their Assay (Husaini et al. 2011)
Evaluation Of Fungal Treated Ankara Fiber(Calotropis procera)
Evaluation of Wood Decay Pattern(Selective or Simultaneous
Light Microscopy
Differential Staining Method using Astra blue and Safranine
1. Weight Loss (Kang et al., 2007)
2. Ash Content (Oluwadare and Asagbra, 2008)
3. Analysis of Chemical Composition of Ankara Fiber:
1%NaOH Solubility (TAPPI, T212 om-02).
Holocellulose content (TAPPI, T 222 om-88).
α-Cellulose content (TAPPI, T 203 cm-99).
Hemicellulose content (Nazarpour et al. 2013).
Lignin Peroxidases
Vertaryl alcohol Assay (at λ310) (Husaini et al., 2011 and Chi et al., 2006)
Manganese Peroxidases
MnSO4 Assay (at λ238) (Husaini et al., 2011 and Chi et al., 2006)
Laccase
DMP Assay(at λ238) (Husaini et al., 2011)
Identification Of The Most Suitable Culture of White Rot Fungi for Biopulping of Ankra (Calotropis procera)

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